‘‘OUTLINE OF THE PROPOSED WORK FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL SCIENCES’’

A SYNOPSIS

“SCREENING AND EVALUATION OF NEUROPROTECTIVE ACTIVITY OF SOME MEDICINAL PLANTS.”

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SCREENING AND EVALUATION OF NEUROPROTECTIVE ACTIVITY OF SOME MEDICINAL PLANTS.

1. AIM & OBJECTIVES:

The present study aims to comparatively evaluate the neuroprotective activity of some medium chain triglycerides of *Cocos nucifera* with below mentioned objectives.

a) To isolate components of coconut oil obtained from fresh meat of varieties of *Cocos nucifera*.
b) To evaluate the neuroprotective activity of some medium chain triglycerides of oil of varieties of *Cocos nucifera* by using colchicine-induced Alzheimer's diseases (AD) in rats.
c) To evaluate the neuroprotective activity of some medium chain triglycerides of oil of varieties of *Cocos nucifera* by using Aluminium chloride-induced AD in rats.
d) To find possible mechanisms of medium chain triglycerides of oil of varieties of *Cocos nucifera* in neuroprotection.

2. INTRODUCTION:

Neurodegeneration is the umbrella term for the progressive loss of structure or function of neurons, including death of neurons. It is estimated that cognitive functions remain relatively stable until an individual reaches approximately 60 years old, at which point they tend to slow down, particularly between 60 and 80 years. There is some evidence that brain function can start to deteriorate as early as 45 years old. In addition, older adults are susceptible to developing neurodegenerative disorders, includes amyotrophic lateral sclerosis, Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegeneration, for which there is no treatment at present. There are many parallels between different neurodegenerative disorders including atypical protein assemblies as well as induced cell death. Discovering these parallels offers hope for therapeutic advances that could ameliorate many diseases simultaneously.

There is currently no known cure, but some available likely candidate such as medium chain triglycerides (MCT) of oil of *Cocos nucifera* (Family- Arecaceae), a source of ketones. It is believed that as ketones are efficiently used by mitochondria for ATP generation and may also help protect vulnerable neurons from free radical damage. Because of these properties, MCT may considered as a protective agent in neurodegeneration.

3. LITERATURE REVIEW:

The extensive literature survey revealed the importance of above mentioned possible therapeutic agents of interest.

Eirikur Benedikz (2009) One of the critical features of an animal model of AD is the ability to analyse memory and cognition in behavioural tests. The differences between the behaviour of rats and mice are far greater than many people realize, although most tasks can be performed by both species. Compared to the rat, the mouse exhibits a simpler behavioural repertoire and much less flexibility in dealing with novel situations. Therefore, the mouse poses a problem for neurobehavioral research as it is a species functioning at a low level of complexity, relative to the rat. Recently, rats have been shown to be able to make adaptive decisions about future behaviour contingent on currently available knowledge. This ability, to reflect on one's own mental processes is termed metacognition and, has previously been thought to be unique to primates. In neuroscience research the rat offers good technological possibilities for neurosurgical/stereotaxic manipulations, neuroimaging, histopathology, electrophysiological recordings or serial sampling of cerebrospinal fluid.[5]

Anil Kumar (2007) The present study concluded that, icv (Intracerebroventricular) colchicine model can be further used to understand better SDAT (sporadic dementia of Alzheimer's type) pathogenesis in humans. It has several features that are consonant with SDAT in humans, including an insidious onset, time dependent changes in behavioral and biochemical patterns. The impairment of working and reference memory, the cardinal symptom of AD and increased oxidative stress, are the hallmarks in the pathophysiology of AD which suggests that icv colchicine administration could be a suitable animal model to study pathophysiology of AD.[6]

Nadia Hichem (2014) The present study investigate the effect of increasing doses of Aluminium chloride ($AlCl_3$) at different periods on body and brain weight, plasma and brain $AlCl_3$ concentration. Histopathological analysis indicates that exposure of rats to $AlCl_3$ causes morphological alterations in dentate gyrus. In fact, $AlCl_3$ had a destructive effect on subgranular layer and granular layer aspect in adult rat. The impairment induced by aluminum was

accompanied primarily by the prolonged accumulation of aluminum in the rat brain. Interestingly, this effect seemed to be different according to dose and duration of exposure.[7]

W. M. A. D. B. Fernando (2015) The lipid content of coconut, being mostly medium chain fatty acid, offers an energy source that bypasses the usual glucose pathway, in the form of ketone bodies, and without the associated fat deposition often caused by light chain fatty acid. Despite the positive effect of a ketogenic diets, whether the ketogenic diets influences beta amyloid levels and protects against AD requires further study. The dosage of ketones and the duration relevant to the AD also needs to be investigated. At this time, it is not clear whether ketone bodies produced from coconut oil has a direct effect on AD, specifically in relation to slowing or clearance of amyloid beta and tau pathologies –and if so, under what conditions. Furthermore, research needs to be conducted to quantify the yield of ketones from virgin coconut oil, and support the ability of coconut derivatives to cross the BBB, to establish likely efficacy.[8]

4. RATIONALE:

All over the world life expectancy has risen, leading to a sharp rise in the number of older persons. While the numbers have gone up, quality of life has gone down. A lot of people see dementia as a natural consequence of getting older, but although there is a strong connection between age and dementia, dementia is a disease, not an age-related defect. AD is the most common cause of dementia. Recent advances in the understanding of AD pathogenesis have led to the development of numerous compounds that might ameliorate the disease process. Research in the field of Alzheimer's disease therapy has been partly successful in terms of developing symptomatic treatments, but also had several failures in terms of developing disease-modifying therapies. Preclinical research is constantly providing new information on pieces of the complex AD puzzle, and an analysis of this information might reveal patterns of pharmacological interactions instead of single potential drug targets. The collaboration between preclinical research, and clinical research has the potential to develop an optimum pharmaceutical approach for the treatment of Alzheimer's disease.

5. PLAN OF WORK:

5.1 Experimental:

5.1.1 Extraction of fresh meat of varieties of *Cocos nucifera*

A) Isolation of oil of varieties of *Cocos nucifera*:

Cold Pressed Coconut Oil[09]- Once coconuts become mature the meat inside becomes hard and thick. The fibrous outer husk has to be removed as well as the hard shell to reveal the hard thick white coconut meat inside. Grate the thick coconut meat into fine shreds. A small amount of good quality water has to be mixed with the finely grated coconut shreds. This water allows for more of the oils to be extracted from the grated coconut when it is pressed.

The coconut shreds then packed into a mesh bag and that bag has to be put into the pressing machine. The bag has to be pressed more than once to extract as much of the coconut milk as possible. The dried material left over after pressing is just the fiber of the coconut. This coconut milk is put into a glass container and left to sit out overnight to separate. The cream rises to the top, the oil in the middle and the water at the bottom.

The cream at the top can be skimmed off and the water at the bottom is discarded. The middle layer is the oil, it has to be filtered and sat to achieve a greater clarity and purity. After filtration the oil may have a slight cloudy appearance. This oil is then covered (with a slight opening) and kept it for about 3 days. During this time any water within will evaporate leaving the clear oil.

B) Isolation of MCT of oil of varieties of *Cocos nucifera*: Fractionated coconut oil provides fractions of the whole oil so that its different fatty acids can be separated for specific uses. Specific method of fractionation will be implemented for isolation of components according to its physico-chemical properties.

Fractionation is a separation process in which a certain quantity of a mixture (gas, solid, liquid, suspension or isotope) is divided during a phase transition, into a number of smaller quantities (fractions) in which the composition varies according to a gradient. Fractions are collected based on differences in a specific property of the individual components. A common trait in fractionations is the need to find an optimum between the amount of fractions collected and the desired purity in each fraction. Fractionation makes it possible to isolate more than two

components in a mixture in a single run. This property sets it apart from other separation techniques. Fractionation of components also takes place in column chromatography by a difference in affinity between stationary phase and the mobile phase. In fractional crystallization and fractional freezing, chemical substances are fractionated based on difference in solubility at a given temperature. The isolated components have to be stored in air tight container until used.

5.1.2 Acute Toxicity Studies:

The isolated each MCT of oil of varieties of *Cocos nucifera* to be administered to six Albino Wistar rats (3 females + 3 males) weighing in the range of 100-200 gm, at a dose of 2000 mg/kg. The rats will be critically observed for clinical signs, gross behavioral changes and mortality if any, following the administration of the test components at different time intervals like 30 min, 1hr, 2hr, 4hr, 24hr, 48hr,and 72hr up to a period of 14 days. The study will be carried out according to OECD Guidelines No.423.[10]

5.1.3 *In vivo* studies:

5.1.3.1 Colchicine-induced Alzheimer's disease in rats [11]:

This study will be carried out on adult male Wistar (albino) rats weighing 180–200g. The animals will be housed under standard laboratory conditions with natural light/dark cycle with free access to standard diet and water throughout the experimental period. All the experiments will be held between 9:00 and 17:00 h. Animals to be divided into following groups (each of 10 rats); as follows:

Group I (Control ACSF-injected): included normal healthy rats that received intracerebroventricular (icv) artificial cerebrospinal fluid (ACSF) 5 µl/site/rat.
ACSF constituents in milli mole (mM): 147 NaCl, 2.9 KCl, 1.6 $MgCl_2$, 1.7 $CaCl_2$ and 2.2 dextrose.

Group II (Untreated AD): included rats with induced AD by icv injection of colchicine dissolved in ACSF 15 µg/rat (7.5 µg in 5µl/site), bilaterally into the lateral ventricle using Hamilton microsyringe.[12]

Remaining groups will be decided after isolation of MCT of oil of varieties of *Cocos nucifera.* Doses will be decided after acute toxicity study.

Animals to be anesthetized with thiopental sodium 45 mg/ kg, intraperitoneally (i.p.), then surgery to be perform. After surgery, rats received gentamycin (5 mg/kg, i.p.) to prevent sepsis and to be provided with special care and to be monitored until spontaneous movement to be occurred. Colchicine has to be dissolved in ACSF.

5.1.3.1.1 Behavioral assessment

5.1.3.1.1.1 Assessment of cognitive performance [06, 13-17]

The acquisition and retention of a spatial navigation task to be assessed using Morris water maze. Animals will be trained to swim to a platform in a circular pool (180 cm diameter × 60 cm), filled with water (28 ± 2^0C) to a depth of 40 cm. The pool has to be divided into 4 quadrants by 4 starting points marked on its wall: North, South, East and West (N– S–E–W). A circular platform 9 cm in diameter has to be placed in the pool 2 cm below water level mounted on a column. The platform provided the only escape from water and has to be fixed in the center of one of the 4 quadrants for the duration of experiment. A colored flag has to be placed outside the pool in a fixed position relative to the pool to help the rat to locate the position of the platform hidden below the water.

(a) Maze acquisition phase (training): On day 13 post-icv injection, rats will receive a training session consisting of 4 trials with an interval of 10 min. In all 4 trials, the starting positions to be different. The time taken by a rat to reach the platform will be recorded as initial acquisition latency (IAL) up to a maximum of 120 s (s).

(b) Maze retention phase (testing for retention of the learned task): Following 24 h (day 14) and 8 days (day 21) after IAL, each rat has to be randomly released at any one of the starting points (N–S–E–W) facing the wall of the pool and tested for the retention of the learnt task. The latency to reach the hidden platform on days 14 and 21 has to be recorded and termed as first and second retention latency (1^{st} RL and 2^{nd} RL) respectively.

5.1.3.1.1.2 Assessment of gross behavioral activity[18]:On days 13, 14 and 21 post-icv injection, the gross behavioral, and spontaneous motor activities of rats has to be assessed in open field arena. The open field chamber is a wooden box (72 × 72cm × 36 cm height) with one wall formed of glass and its floor is divided into equal-sized squares. Open field task has to be performed immediately after water maze test.

5.1.3.1.2 Biochemical tests

Next day after the last behavioral test, all rats has to be decapitated under ether anesthesia and the brains has to be rapidly removed and rinsed with ice-cold phosphate buffered saline (PBS) solution, pH7.4 to remove any red blood cells and clots. Then, the hippocampus has to be dissected and the following measurements to be done:

5.1.3.1.2.1 Hippocampal tissue levels of brain derived neurotrophic factor (BDNF)[19]

5.1.3.1.2.2 Hippocampal tissue levels of amyloid beta (Ab) peptide[19]

5.1.3.1.2.3 Hippocampal tissue levels of antioxidant system: glutathione reductase (GR),[20] and glutathione peroxidase (GPX).[21]

5.1.3.1.3 Statistical analysis

The changes in the studied parameters has to be expressed as mean ± SD. Differences between groups has to be compared by one way ANOVA using the statistical software. Statistical significance has to be set at p value less than 0.05.

5.1.3.2 Aluminium chloride-induced Alzheimer's disease in rats[22]**:**

This study will be carried out adult male Wistar (albino) rats weighing 180–200g. The animals will be housed under standard laboratory conditions with natural light/dark cycle with free access to standard diet and water throughout the experimental period. All the experiments will be held between 9:00 and 17:00 h. For oral administration, aluminium chloride has to be dissolved in sterile water, at a dose of 0.5 ml/100 g body weight. Animals to be divided into groups (each of 07 rats); as follows:

Group I (Control): Included normal healthy rats that received sterile water orally at a dose of 0.5 ml/ 100 g of body weight daily for 42 days.

Group II (Untreated AD): Included rats with induced AD by oral administration of aluminium chloride ($AlCl_3$) 100 mg/kg daily for 42 days.

Remaining groups will be decided after isolation of MCT of oil of varieties of *Cocos nucifera*. Doses will be decided after acute toxicity study.

5.1.3.2.1 Behavioral assessment

5.1.3.2.1.1 Assessment of cognitive performance [23]

The acquisition and retention of a spatial navigation task to be assessed using Morris water maze. Animals will be trained to swim to a platform in a circular pool (180 cm diameter × 60 cm), filled with water (28 ± 2^0C) to a depth of 40 cm. The pool has to be divided into 4 quadrants by 4 starting points marked on its wall: North, South, East and West (N– S–E–W). A circular platform 9 cm in diameter has to be placed in the pool 2 cm below water level mounted on a column. The platform provided the only escape from water and has to be fixed in the center of one of the 4 quadrants for the duration of experiment. A colored flag has to be placed outside the pool in a fixed position relative to the pool to help the rat to locate the position of the platform hidden below the water.

(a) Maze acquisition phase (training): Animals received two consecutive daily training sessions on day 19 and 20. During the acquisition phase, each rat has to be put into the water in any one of four starting positions, the sequence of which has to be selected randomly. The latency to reach the platform (acquisition latency) has to be measured.

(b) Maze retention phase (testing for retention of the learned task): Twenty-four hours (day 21) and 21 days (day 42) after the acquisition phase, each animal to be released randomly from one of the edges facing the wall of the pool to assess memory retention. Time latency to find the hidden platform on day 21 and 42 following the start of aluminium chloride administration has to be recorded and termed the first retention latency (1^{st} RL) (21^{st} day) and the second retention latency (2^{nd} RL)(42^{nd} day), respectively.

5.1.3.2.1.2 Assessment of gross behavioral activity[18]: On days 21 and 42 following the start of aluminium chloride administration, the gross behavioral, and spontaneous motor activities of rats has to be assessed in open field arena. The open field chamber is a wooden box (72 × 72cm × 36 cm height) with one wall formed of glass and its floor is divided into equal-sized squares. Open field task has to be performed immediately after water maze test.

5.1.3.2.2 Biochemical tests

Next day after the last behavioral test, all rats has to be decapitated under ether anesthesia and brains has to be removed and rinsed with ice-cold isotonic saline and homogenized with ice-cold 0.1 mmol/l phosphate buffer (pH 7.4). The homogenates (10% w/v) has to be then centrifuged at 10,000 × g for 15 min and the supernatant so formed has to be used for the biochemical analysis.

5.1.3.2.2.1 Measurement of lipid peroxidation[24]

5.1.3.2.2.2 Estimation of nitrite[25]

5.1.3.2.2.3 Estimation of reduced glutathione[26]

5.1.3.2.2.4 Superoxide dismutase activity[27]

5.1.3.2.2.5 Catalase activity[28]

5.1.3.2.2.6 Glutathione S-transferase activity[29]

5.1.3.2.2.7 Estimation of acetylcholinesterase (AChE) Activity[30]

5.1.3.2.2.8 Protein estimation[31]

5.1.3.2.2.9 Aluminium estimation[32]

5.1.3.2.3 Statistical analysis

Values are expressed as the mean ± SEM. The behavioural assessment data has to be analyzed by a repeated measures two-way analysis of variance (ANOVA). The biochemical estimations have to be separately analyzed by one-way ANOVA. $p < 0.05$ has to be considered significant.

6. DURATION OF WORK:

- Screening and isolation of MCT of varieties *Cocos nucifera*.
- The neuroprotective activity of MCT of varieties of *Cocos nucifera* will be evaluated by using colchicine induced Alzheimer's disease in rats.
- The neuroprotective activity of MCT of varieties of *Cocos nucifera* will be evaluated by using Aluminium chloride induced Alzheimer's disease in rats.

Sr. No.	Activity	Tentative Duration (months)
1	Literature Review.	06
2	Selection and procurement of drugs and chemicals.	06
3	Screening and isolation of MCT of oil of varieties of *Cocos nucifera*.	06
4	Pharmacological screening of MCT of oil of varieties of *Cocos nucifera*.	12
5	Compilation, interpretation of data and writing of thesis.	06
	Total	36

7. RESEARCH OUTCOME:

The biggest achievement of the century is longevity, which may get contributed with selection of most possible treatments for unpredictable disease like AD. When a natural protein on the surface of nerve cells is destroyed by enzymes in fragments, called beta-amyloid, clump together, and this makes nerve cells stop working. Nerve cells are also destroyed from within because the tau proteins that keep the cells together begin to tangle with the result that the cells die. The present study will be carried out based on the hypothesis related with the research topic. Therefore, chances of getting breakthrough are more, which may be patented or published in highly, reputed journals.

8. REFERENCES:

1. Singh-Manoux A. et al. Timing of onset of cognitive decline: results from Whitehall II prospective cohort study. *BMJ*, 344 Available at: doi:http//dx.doi.org/10.1136/bmj.d7622 (2012).
2. Alzheimer Europe, 'Alzheimer's disease. Available at: http://www.alzheimer-europe.org/Dementia/Alzheimer-s-disease (2015).
3. Mathuranath PS et al, Incidence of Alzheimer's disease in India: a 10 years follow-up study, Neurology India. 60(6), 625-630, (2012).
4. CNB.COM on http://www.cbn.com/media/player/index.aspx?s=/mp4/LJO190v1_WS.
5. Eirikur Benedikz et al, The rat as an animal model of Alzheimer's disease, Journal *of* Cellular *and* Molecular Medicine. 13*(*6), 1034-1042, (2009).
6. Anil Kumar et al, Colchicines-induced neurotoxicity as an animal model of sporadic dementia of Alzheimer's type, Pharmacological reports. 59, 274-283, (2007).
7. Nadia Hichem et al, Aluminum chloride impacts dentate gyrus structure in male adult albino wistar rats, Tissue and Cell. 46 (6), 409-414, (2014).
8. Fernando WM et al, The role of dietary coconut for the prevention and treatment of Alzheimer's disease: potential mechanisms of action, British Journal of Nutrition. 114(1), 1-14, (2015).
9. http://www.making-healthy-choices.com/coconut-oil-benefits.html
10. OECD Guideline For Testing Of Chemicals Acute Oral Toxicity – Acute Toxic Class Method (2001).
11. Adham R. et al, Neuroprotective role of vitamin D3 in colchicine-induced Alzheimer's disease in rats, Alexandria Journal of Medicine. 51, 127-136, (2015).
12. Kumar Veerenda MH et al, Intracerebroventricular administration of colchicine produces cognitive impairment associated with oxidative stress in rats, Pharmacology Biochemistry and Behavior. 73, 565-571, (2002).

13. Torres LL et al, Peripheral oxidative stress biomarkers in mild cognitive impairment and Alzheimer's disease, Journal of Alzheimer's Disease. 26(1), 59-68, (2011).

14. Hardy J et al, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science, 297, 353-356, (2002).

15. Shiiki T et al, Brain insulin impairs amyloid- beta clearance from the brain, The Journal of Neuroscience. 24(43), 9632-9637, (2004).

16. Llewellyn DJ et al, Serum 25-hydroxyvitamin D concentration and cognitive impairment, Journal of Geriatric Psychiatry and Neurology. 22 (3), 188-95 (2009).

17. Kumar A et al, Neuroprotective effect of Centella Asiatica against intracerebroventricular colchicine-induced cognitive impairment and oxidative stress, International Journal of Alzheimer's Disease. Article ID 972178, 8 pages, (2009).

18. Jahkel M et al, Open field locomotion and neurotransmission in mice evaluated by principal component factor analysis-effects of housing condition, individual activity disposition and psychotropic drugs, Progress in Neuro-Psychopharmacology and Biological Psychiatry. 24 (1), 61-84, (2000).

19. Lowry OH et al, Protein measurement with the folin phenol reagent, The journal of biological chemistry. 193, 265-275, (1951).

20. Carberg I et al, Glutathione reductase levels in rat brain, The journal of biological chemistry. 250, 5475-5480, (1975).

21. Wheeler CR et al, Automated assay for superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activity, Analytical Biochemistry. 184 (2), 193-199 (1990).

22. Kumar A et al, Neuroprotective effect of carvedilol against aluminium induced toxicity: possible behavioural and biochemical alterations in rats, Pharmacological reports. 63, 915-923, (2011).

23. Prakash A et al, Effect of N-acetyl cysteine against aluminium-induced cognitive dysfunction and oxidative damage in rats, Basic and Clinical Pharmacology and Toxicology. 105, 98-104, (2009).

24. Wills ED, Mechanism of lipid peroxide formation in animal tissues, Journal of Biochemistry. 99, (3), 667-676, (1966).

25. Green LC et al, Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids, Analytical Biochemistry. 126 (1), 131-138, (1982).

26. Ellman GL, Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics. 82 (1), 70-77, (1959).

27. Kono Y, Generation of superoxide radical during autooxidation of hydroxylamine and an assay for superoxide dismutase, Archives of Biochemistry and Biophysics. 186 (1), 189-195, (1978).

28. Luck H, Catalase, Methods of Enzymatic Analysis. 885-894, (1965).

29. Habig WH et al, Assays for differentiation of glutathione-S-transferases, Methods of Enzymology. 77, 398-405 (1981).

30. Ellman GL et al, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochemical Pharmacology. 7, 88-95, (1961).

31. Gornall AG et al, Determination of serum proteins by means of biuret reaction, The Journal of Biological Chemistry. 177 (2), 751-766, (1949).

32. Zumkley H et al, Aluminium, Zinc and Copper concentrations in plasma in chronic renal insufficiency, Clinical Nephrology. 12 (1), 18-21, (1979).

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